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(54) Title: RORs AS MODIFIERS OF THE p21 PATHWAY AND METHODS OF USE

(57) Abstract: Human ROR genes are identified as modulators of the p21 pathway, and thus are therapeutic targets for disorders associated with defective p21 function. Methods for identifying modulators of p21, comprising screening for agents that modulate the activity of ROR are provided.

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## RORs AS MODIFIERS OF THE p21 PATHWAY AND METHODS OF USE

### REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional patent application 60/411,010  
5 filed 9/16/2002. The contents of the prior application are hereby incorporated in their entirety.

### BACKGROUND OF THE INVENTION

The p21/CDKN1/WAF1/CIP1 protein(El-Deiry, W. S.; et al. Cell 75: 817-825,  
10 1993; Harper, J. W.; et al. Cell 75: 805-816, 1993; Huppi, Ket al. Oncogene 9: 3017-3020, 1994) is a cell cycle control protein that inhibits cyclin-kinase activity, is tightly regulated at the transcriptional level by p53, and mediates p53 suppression of tumor cell growth. Along with p53, p21 appears to be essential for maintaining the G2 checkpoint in human cells (Bunz, F.; Dutriau, A.; et al. Science 282:1497-1501, 1998). Sequences of P21 are  
15 well-conserved throughout evolution, and have been identified in species as diverse as human (Genbank Identifier 13643057), *Drosophila melanogaster* (GI# 1684911), *Caenorhabditis elegans* (GI#4966283), and yeast (GI#2656016).

RORA (retinoic acid receptor related orphan receptor A) is a member of the nuclear hormone receptor superfamily (Giguere, V., et al (1994) Genes And Development  
20 8: 538-53). Members of this gene family include the steroid hormone, thyroid hormone and retinoid receptors, and orphan receptors for which a ligand has not yet been identified. Members of this superfamily also share a common modular structure composed of a transactivation domain, a DNA-binding domain, and a ligand-binding domain. Typically, their transcriptional transactivation function is regulated by small lipophilic molecules,  
25 such as steroid hormones, vitamin D, retinoic acids, and thyroid hormone. These molecules are synthesized in the organism and pass readily through the plasma membrane to reach the corresponding receptors inside the cell.

Mutations in the RORA gene have been related to recessive Robinow syndrome (Afzal, A. R., et al (2000) Nat Genet 25:419-22). RORB is a transcription factor and  
30 interacts with NM23-2, a nucleoside diphosphate kinase involved in organogenesis and differentiation (Paravicini, G. et al. (1996) Biochem. Biophys. Res. Commun. 227: 82-87). RORC (ROR-gamma) is important for lymphoid organogenesis and plays an important regulatory role in thymopoiesis (Kurebayashi, S. et al (2000) Proc. Nat. Acad. Sci. 97: 10132-10137).

The ability to manipulate the genomes of model organisms such as *Drosophila* provides a powerful means to analyze biochemical processes that, due to significant evolutionary conservation, have direct relevance to more complex vertebrate organisms. Due to a high level of gene and pathway conservation, the strong similarity of cellular processes, and the functional conservation of genes between these model organisms and mammals, identification of the involvement of novel genes in particular pathways and their functions in such model organisms can directly contribute to the understanding of the correlative pathways and methods of modulating them in mammals (see, for example, Mechler BM et al., 1985 EMBO J 4:1551-1557; Gateff E. 1982 Adv. Cancer Res. 37: 33-74; Watson KL., et al., 1994 J Cell Sci. 18: 19-33; Miklos GL, and Rubin GM. 1996 Cell 86:521-529; Wassarman DA, et al., 1995 Curr Opin Gen Dev 5: 44-50; and Booth DR. 1999 Cancer Metastasis Rev. 18: 261-284). For example, a genetic screen can be carried out in an invertebrate model organism having underexpression (e.g. knockout) or overexpression of a gene (referred to as a "genetic entry point") that yields a visible phenotype. Additional genes are mutated in a random or targeted manner. When a gene mutation changes the original phenotype caused by the mutation in the genetic entry point, the gene is identified as a "modifier" involved in the same or overlapping pathway as the genetic entry point. When the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as p21, modifier genes can be identified that may be attractive candidate targets for novel therapeutics.

All references cited herein, including patents, patent applications, publications, and sequence information in referenced Genbank identifier numbers, are incorporated herein in their entireties.

## SUMMARY OF THE INVENTION

We have discovered genes that modify the p21 pathway in *Drosophila*, and identified their human orthologs, hereinafter referred to as retinoic acid receptor related orphan receptor (ROR). The invention provides methods for utilizing these p21 modifier genes and polypeptides to identify ROR-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired p21 function and/or ROR function. Preferred ROR-modulating agents specifically bind to ROR polypeptides and restore p21 function. Other preferred ROR-modulating agents are nucleic acid modulators such as antisense oligomers and RNAi that repress ROR gene

expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

ROR modulating agents may be evaluated by any convenient *in vitro* or *in vivo* assay for molecular interaction with an ROR polypeptide or nucleic acid. In one embodiment, candidate ROR modulating agents are tested with an assay system comprising a ROR polypeptide or nucleic acid. Agents that produce a change in the activity of the assay system relative to controls are identified as candidate p21 modulating agents. The assay system may be cell-based or cell-free. ROR-modulating agents include ROR related proteins (e.g. dominant negative mutants, and biotherapeutics); ROR - specific antibodies; ROR -specific antisense oligomers and other nucleic acid modulators; and chemical agents that specifically bind to or interact with ROR or compete with ROR binding partner (e.g. by binding to an ROR binding partner). In one specific embodiment, a small molecule modulator is identified using a binding assay. In specific embodiments, the screening assay system is selected from an apoptosis assay, a cell proliferation assay, an angiogenesis assay, and a hypoxic induction assay.

In another embodiment, candidate p21 pathway modulating agents are further tested using a second assay system that detects changes in the p21 pathway, such as angiogenic, apoptotic, or cell proliferation changes produced by the originally identified candidate agent or an agent derived from the original agent. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating the p21 pathway, such as an angiogenic, apoptotic, or cell proliferation disorder (e.g. cancer).

The invention further provides methods for modulating the ROR function and/or the p21 pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a ROR polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to a mammalian animal predetermined to have a pathology associated the p21 pathway.

## DETAILED DESCRIPTION OF THE INVENTION

A dominant loss of function screen was carried out in *Drosophila* to identify genes that interact with the cyclin dependent kinase inhibitor, p21 (Bourne HR, et al., Nature (1990) 348(6297):125-132; Marshall CJ, Trends Genet (1991) 7(3):91-95). Expression of the p21 gene in the eye causes deterioration of normal eye morphology. Modifiers of the

eye phenotype were identified as members of the p21 pathway. The HR46 gene was identified as a modifier of the p21 pathway. Accordingly, vertebrate orthologs of these modifiers, and preferably the human orthologs, ROR genes (i.e., nucleic acids and polypeptides) are attractive drug targets for the treatment of pathologies associated with a defective p21 signaling pathway, such as cancer.

In vitro and in vivo methods of assessing ROR function are provided herein. Modulation of the ROR or their respective binding partners is useful for understanding the association of the p21 pathway and its members in normal and disease conditions and for developing diagnostics and therapeutic modalities for p21 related pathologies. ROR-modulating agents that act by inhibiting or enhancing ROR expression, directly or indirectly, for example, by affecting an ROR function such as binding activity, can be identified using methods provided herein. ROR modulating agents are useful in diagnosis, therapy and pharmaceutical development.

#### **15 Nucleic acids and polypeptides of the invention**

Sequences related to ROR nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) number) as GI#s 19743899 (SEQ ID NO:1), 19743900 (SEQ ID NO:2), 19743902 (SEQ ID NO:3), 451565 (SEQ ID NO:4), 14250723 (SEQ ID NO:5), 348240 (SEQ ID NO:6), 19743904 (SEQ ID NO:7), 19743906 (SEQ ID NO:8), 1619293 (SEQ ID NO:9), 30704550 (SEQ ID NO:10), 19743908 (SEQ ID NO:11), 21739736 (SEQ ID NO:12), 758419 (SEQ ID NO:13), 21594879 (SEQ ID NO:14), and 21757912 (SEQ ID NO:15) for nucleic acid, and GI#s 19743901 (SEQ ID NO:16), 19743907 (SEQ ID NO:17), and 19743909 (SEQ ID NO:18) for polypeptides.

The term "ROR polypeptide" refers to a full-length ROR protein or a functionally active fragment or derivative thereof. A "functionally active" ROR fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type ROR protein, such as antigenic or immunogenic activity, ability to bind natural cellular substrates, etc. The functional activity of ROR proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey) and as further discussed below. In one embodiment, a functionally active ROR polypeptide is a ROR derivative capable of rescuing defective endogenous ROR activity, such as in cell based or animal assays; the rescuing derivative may be from the same or a different

species. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of an ROR, such as a binding domain.

Protein domains can be identified using the PFAM program (Bateman A., et al., *Nucleic Acids Res*, 1999, 27:260-2). For example, the zinc finger domain (PFAM 00105) of ROR from GI#s 19743901, 19743907, and 19743909 (SEQ ID NOs:16, 17, and 18, respectively) is located respectively at approximately amino acid residues 104 to 179, 8 to 83, and 29 to 104. Likewise, the Ligand-binding domain of nuclear hormone receptor domain (PFAM 00104) of SEQ ID NOs:16, 17, and 18 is located respectively at approximately amino acid residues 361 to 544, 267 to 450, and 325 to 506. Methods for obtaining ROR polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of any one of SEQ ID NOs:16-18 (an ROR). In further preferred embodiments, the fragment comprises the entire functionally active domain.

The term "ROR nucleic acid" refers to a DNA or RNA molecule that encodes a ROR polypeptide. Preferably, the ROR polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with human ROR. Methods of identifying orthologs are known in the art. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, *Proc Natl Acad Sci* (1998) 95:5849-5856; Huynen MA *et al.*, *Genome Research* (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994, *Nucleic Acids Res* 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences,

Salzburg, Austria) may also identify potential orthologs. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *Drosophila*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, Advances in Applied Mathematics 2:482-489; database: European Bioinformatics Institute; Smith and Waterman, 1981, J. of Molec.Biol., 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.; W.R. Pearson, 1991, Genomics 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff

(Dayhoff: Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 Nucl. Acids Res. 14(6):6745-6763). The Smith-Waterman algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated, the "Match" value reflects "sequence identity."

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of any of SEQ ID NOs:1-15. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of any one of SEQ ID NOs:1-15 under high stringency hybridization conditions that are: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 µg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate).

In other embodiments, moderately stringent hybridization conditions are used that are: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Alternatively, low stringency conditions can be used that are: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 µg/ml



denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

### **Isolation, Production, Expression, and Mis-expression of ROR Nucleic Acids and Polypeptides**

ROR nucleic acids and polypeptides are useful for identifying and testing agents that modulate ROR function and for other applications related to the involvement of ROR in the p21 pathway. ROR nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods. For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of an ROR protein for assays used to assess ROR function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (*e.g.*, Higgins SJ and Hames BD (eds.) *Protein Expression: A Practical Approach*, Oxford University Press Inc., New York 1999; Stanbury PF et al., *Principles of Fermentation Technology*, 2<sup>nd</sup> edition, Elsevier Science, New York, 1995; Doonan S (ed.) *Protein Purification Protocols*, Humana Press, New Jersey, 1996; Coligan JE et al, *Current Protocols in Protein Science* (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant ROR is expressed in a cell line known to have defective p21 function such as HCT116 colon cancer cells available from American Type Culture Collection (ATCC), Manassas, VA). The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

The nucleotide sequence encoding an ROR polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native ROR gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may

be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. An isolated host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

To detect expression of the ROR gene product, the expression vector can comprise a promoter operably linked to an ROR gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the ROR gene product based on the physical or functional properties of the ROR protein in *in vitro* assay systems (*e.g.* immunoassays).

The ROR protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (*i.e.* it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

Once a recombinant cell that expresses the ROR gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). Alternatively, native ROR proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of ROR or other genes associated with the p21 pathway. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

### **Genetically modified animals**

Animal models that have been genetically modified to alter ROR expression may be used in *in vivo* assays to test for activity of a candidate p21 modulating agent, or to

further assess the role of ROR in a p21 pathway process such as apoptosis or cell proliferation. Preferably, the altered ROR expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal ROR expression. The genetically modified animal may additionally have altered p21 expression (e.g. p21 knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice or rats), among others. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4:761-763.) or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al., and Hogan, B., Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sanford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, Science (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et al.*, A Universal Marker for Transgenic Insects (1999) Nature 402:370-371; for transgenic Zebrafish see Lin S., Transgenic Zebrafish, Methods Mol Biol. (2000);136:375-3830); for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, Experientia (1991) 47:897-905; for transgenic rats see Hammer *et al.*, Cell (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) Nature 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous ROR gene that

results in a decrease of ROR function, preferably such that ROR expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse ROR gene is used to construct a homologous recombination vector suitable for altering an endogenous ROR gene in the mouse genome. Detailed methodologies for homologous recombination in mice are available (see Capecchi, Science (1989) 244:1288-1292; Joyner *et al.*, Nature (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, Science (1989) 244:1281-1288; Simms *et al.*, Bio/Technology (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene that has been knocked out (Claesson MH *et al.*, (1994) Scan J Immunol 40:257-264; Declerck PJ *et al.*, (1995) J Biol Chem. 270:8397-400).

In another embodiment, the transgenic animal is a "knock-in" animal having an alteration in its genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the ROR gene, e.g., by introduction of additional copies of ROR, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the ROR gene. Such regulatory sequences include inducible, tissue-specific, and constitutive promoters and enhancer elements. The knock-in can be homozygous or heterozygous.

Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*, PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.*

(1991) Science 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the transgene, and for sequential deletion of vector sequences in the same cell (Sun X et al (2000) Nat Genet 25:83-6).

5           The genetically modified animals can be used in genetic studies to further elucidate the p21 pathway, as animal models of disease and disorders implicating defective p21 function, and for *in vivo* testing of candidate therapeutic agents, such as those identified in screens described below. The candidate therapeutic agents are administered to a genetically modified animal having altered ROR function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that receive placebo treatment, and/or animals with unaltered ROR expression that receive candidate therapeutic agent.

10           In addition to the above-described genetically modified animals having altered ROR function, animal models having defective p21 function (and otherwise normal ROR function), can be used in the methods of the present invention. For example, a p21 knockout mouse can be used to assess, *in vivo*, the activity of a candidate p21 modulating agent identified in one of the *in vitro* assays described below. p21 knockout mice are described in the literature (Umanoff H, et al., Proc Natl Acad Sci U S A 1995 Feb 28;92(5):1709-13). Preferably, the candidate p21 modulating agent when administered to a model system with cells defective in p21 function, produces a detectable phenotypic change in the model system indicating that the p21 function is restored, i.e., the cells exhibit normal cell cycle progression.

### **Modulating Agents**

25           The invention provides methods to identify agents that interact with and/or modulate the function of ROR and/or the p21 pathway. Modulating agents identified by the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the p21 pathway, as well as in further analysis of the ROR protein and its contribution to the p21 pathway. Accordingly, the invention also provides methods for modulating the p21 pathway comprising the step of specifically modulating ROR activity by administering a ROR-interacting or -modulating agent.

As used herein, an "ROR-modulating agent" is any agent that modulates ROR function, for example, an agent that interacts with ROR to inhibit or enhance ROR activity

or otherwise affect normal ROR function. ROR function can be affected at any level, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a preferred embodiment, the ROR - modulating agent specifically modulates the function of the ROR. The phrases "specific modulating agent",

5 "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the ROR polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the ROR. These phrases also encompass modulating agents that alter the interaction of the ROR with a binding partner, substrate, or cofactor (e.g. by binding to a binding partner of an ROR, or to a protein/binding partner complex, and altering ROR  
10 function). In a further preferred embodiment, the ROR- modulating agent is a modulator of the p21 pathway (e.g. it restores and/or upregulates p21 function) and thus is also a p21-modulating agent.

Preferred ROR-modulating agents include small molecule compounds; ROR-interacting proteins, including antibodies and other biotherapeutics; and nucleic acid  
15 modulators such as antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19<sup>th</sup>  
20 edition.

#### **Small molecule modulators**

Small molecules are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents,  
25 referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more preferably less than 1,000, and most preferably less than 500 daltons. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or  
30 identified based on known or inferred properties of the ROR protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for ROR-modulating activity. Methods for generating and obtaining compounds are well known in the art

(Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the p21 pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

### Protein Modulators

Specific ROR-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to the p21 pathway and related disorders, as well as in validation assays for other ROR-modulating agents. In a preferred embodiment, ROR-interacting proteins affect normal ROR function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, ROR-interacting proteins are useful in detecting and providing information about the function of ROR proteins, as is relevant to p21 related disorders, such as cancer (e.g., for diagnostic means).

An ROR-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with an ROR, such as a member of the ROR pathway that modulates ROR expression, localization, and/or activity. ROR-modulators include dominant negative forms of ROR-interacting proteins and of ROR proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous ROR-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3<sup>rd</sup>, Trends Genet (2000) 16:5-8).

An ROR-interacting protein may be an exogenous protein, such as an ROR-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory; Harlow and Lane (1999) *Using antibodies: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). ROR antibodies are further discussed below.

In preferred embodiments, an ROR-interacting protein specifically binds an ROR protein. In alternative preferred embodiments, an ROR-modulating agent binds an ROR substrate, binding partner, or cofactor.

### *Antibodies*

In another embodiment, the protein modulator is an ROR specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify ROR modulators. The antibodies can also be used in dissecting the portions of the ROR pathway responsible for various cellular responses and in the general processing and maturation of the ROR.

Antibodies that specifically bind ROR polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of ROR polypeptide, and more preferably, to human ROR. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Epitopes of ROR which are particularly antigenic can be selected, for example, by routine screening of ROR polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), *Proc. Natl. Acad. Sci. U.S.A.* 78:3824-28; Hopp and Wood, (1983) *Mol. Immunol.* 20:483-89; Sutcliffe et al., (1983) *Science* 219:660-66) to the amino acid sequence shown in any of SEQ ID NOs:16-18. Monoclonal antibodies with affinities of  $10^8 \text{ M}^{-1}$  preferably  $10^9 \text{ M}^{-1}$  to  $10^{10} \text{ M}^{-1}$ , or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of ROR or substantially purified fragments thereof. If ROR fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of an ROR protein. In a particular embodiment, ROR-specific antigens and/or immunogens are coupled to carrier proteins that stimulate the



immune response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

5       The presence of ROR-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding ROR polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

10       Chimeric antibodies specific to ROR polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al.,  
15       Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant  
20       DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 10:239-265). Humanized antibodies and methods of their production are well-known in  
25       the art (U.S. Pat. Nos. 5,530,101, 5,585,089, 5,693,762, and 6,180,370).

      ROR-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No. 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA  
30       (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

      Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As

used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989) 4:131-134). A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg –to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml to about 10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

### ***Specific biotherapeutics***

In a preferred embodiment, an ROR-interacting protein may have biotherapeutic applications. Biotherapeutic agents formulated in pharmaceutically acceptable carriers

and dosages may be used to activate or inhibit signal transduction pathways. This modulation may be accomplished by binding a ligand, thus inhibiting the activity of the pathway; or by binding a receptor, either to inhibit activation of, or to activate, the receptor. Alternatively, the biotherapeutic may itself be a ligand capable of activating or inhibiting a receptor. Biotherapeutic agents and methods of producing them are described in detail in U.S. Pat. No. 6,146,628.

ROR, its ligand(s), antibodies to the ligand(s) or the ROR itself may be used as biotherapeutics to modulate the activity of ROR in the p21 pathway.

### **Nucleic Acid Modulators**

Other preferred ROR-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit ROR activity. Preferred nucleic acid modulators interfere with the function of the ROR nucleic acid such as DNA replication, transcription, translocation of the ROR RNA to the site of protein translation, translation of protein from the ROR RNA, splicing of the ROR RNA to yield one or more mRNA species, or catalytic activity which may be engaged in or facilitated by the ROR RNA.

In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to an ROR mRNA to bind to and prevent translation, preferably by binding to the 5' untranslated region. ROR-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

In another embodiment, the antisense oligomer is a phosphothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits, each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphodiamidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense

Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. :7:187-95; US Pat. No. 5,235,033; and US Pat No. 5,378,841).

Alternative preferred ROR nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498).

Nucleic acid modulators are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Nucleic acid modulators are also used, for example, to distinguish between functions of various members of a biological pathway. For example, antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, an ROR-specific nucleic acid modulator is used in an assay to further elucidate the role of the ROR in the p21 pathway, and/or its relationship to other members of the pathway. In another aspect of the invention, an ROR-specific antisense oligomer is used as a therapeutic agent for treatment of p21-related disease states.

### Assay Systems

The invention provides assay systems and screening methods for identifying specific modulators of ROR activity. As used herein, an "assay system" encompasses all

the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the ROR nucleic acid or protein. In general, secondary assays further assess the activity of a ROR modulating agent identified by a primary assay and may confirm that the modulating agent affects ROR in a manner relevant to the p21 pathway. In some cases, ROR modulators will be directly tested in a secondary assay.

In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising an ROR polypeptide or nucleic acid with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. binding activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates ROR activity, and hence the p21 pathway. The ROR polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

### Primary Assays

The type of modulator tested generally determines the type of primary assay.

#### *Primary assays for small molecule modulators*

For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (e.g., receptor-ligand binding), transcriptional activity (e.g., using a reporter gene), enzymatic activity (e.g., via a property of the substrate), activity of second messengers, immunogenicity and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent, radioactive, colorimetric,

spectrophotometric, and amperometric methods, to provide a read-out for the particular molecular event detected.

Cell-based screening assays usually require systems for recombinant expression of ROR and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when ROR-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the ROR protein may be assayed by various known methods such as substrate processing (e.g. ability of the candidate ROR-specific binding agents to function as negative effectors in ROR-expressing cells), binding equilibrium constants (usually at least about  $10^7 \text{ M}^{-1}$ , preferably at least about  $10^8 \text{ M}^{-1}$ , more preferably at least about  $10^9 \text{ M}^{-1}$ ), and immunogenicity (e.g. ability to elicit ROR specific antibody in a heterologous host such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of a ROR polypeptide, a fusion protein thereof, or to cells or membranes bearing the polypeptide or fusion protein. The ROR polypeptide can be full length or a fragment thereof that retains functional ROR activity. The ROR polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The ROR polypeptide is preferably human ROR, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay detects candidate agent-based modulation of ROR interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has ROR-specific binding activity, and can be used to assess normal ROR gene function.

Suitable assay formats that may be adapted to screen for ROR modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, Curr Opin Chem Biol (1998) 2:597-603; Sundberg SA, Curr Opin Biotechnol 2000, 11:47-53). In one preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to

monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, Nat Struct Biol (2000) 7:730-4; Fernandes PB, *supra*; Hertzberg RP and Pope AJ, Curr Opin Chem Biol (2000) 4:445-451).

5 A variety of suitable assay systems may be used to identify candidate ROR and p21 pathway modulators (*e.g.* U.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); and U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434 (angiogenesis assays), among others). Specific preferred assays are described in more detail below.

10 Nuclear receptors (NRs) are a superfamily of ligand-dependent transcription factors that mediate the effects of hormones and other endogenous ligands to regulate the expression of specific genes. High throughput assays for nuclear receptors include fluorescent polarization binding assays (Lin S, et al. (2002) Anal Biochem 300(1):15-21), and homogeneous time-resolved fluorescence energy transfer (Zhou G, et al. (2001) Methods 25:54-61), among others.

15 **Apoptosis assays.** Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of  
20 fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., et al., 1998, Blood 15:4730-41). Other cell-based apoptosis assays include the caspase-3/7 assay and the cell death nucleosome ELISA assay. The caspase 3/7 assay is based on the activation of the caspase cleavage activity as part of a cascade of events that occur during programmed cell  
25 death in many apoptotic pathways. In the caspase 3/7 assay (commercially available Apo-ONE™ Homogeneous Caspase-3/7 assay from Promega, cat# 67790), lysis buffer and caspase substrate are mixed and added to cells. The caspase substrate becomes fluorescent when cleaved by active caspase 3/7. The nucleosome ELISA assay is a general cell death assay known to those skilled in the art, and available commercially (Roche, Cat#  
30 1774425). This assay is a quantitative sandwich-enzyme-immunoassay which uses monoclonal antibodies directed against DNA and histones respectively, thus specifically determining amount of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Mono and oligonucleosomes are enriched in the cytoplasm during apoptosis due to the fact that DNA fragmentation occurs several hours before the plasma membrane

breaks down, allowing for accumulation in the cytoplasm. Nucleosomes are not present in the cytoplasmic fraction of cells that are not undergoing apoptosis. An apoptosis assay system may comprise a cell that expresses an ROR, and that optionally has defective p21 function (e.g. p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate p21 modulating agents. In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate p21 modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether ROR function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express ROR relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the ROR plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

**Cell proliferation and cell cycle assays.** Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, *Int. J. Cancer* 38, 369; Campana *et al.*, 1988, *J. Immunol. Meth.* 107, 79), or by other means.

Cell proliferation is also assayed via phospho-histone H3 staining, which identifies a cell population undergoing mitosis by phosphorylation of histone H3. Phosphorylation of histone H3 at serine 10 is detected using an antibody specific to the phosphorylated form of the serine 10 residue of histone H3. (Chadlee, D.N. 1995, *J. Biol. Chem* 270:20098-105). Cell Proliferation may also be examined using [<sup>3</sup>H]-thymidine incorporation (Chen, J., 1996, *Oncogene* 13:1395-403; Jeoung, J., 1995, *J. Biol. Chem.* 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [<sup>3</sup>H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter). Another proliferation assay uses the dye Alamar Blue (available from Biosource International), which fluoresces when reduced in living cells and provides an indirect measurement of cell number (Voytik-Harbin SL *et al.*, 1998, *In Vitro Cell Dev Biol Anim* 34:239-46). Yet another proliferation assay, the MTS assay, is based on in



vitro cytotoxicity assessment of industrial chemicals, and uses the soluble tetrazolium salt, MTS. MTS assays are commercially available, for example, the Promega CellTiter 96<sup>®</sup> Aqueous Non-Radioactive Cell Proliferation Assay (Cat.# G5421).

5 Cell proliferation may also be assayed by colony formation in soft agar (Sambrook et al., Molecular Cloning, Cold Spring Harbor (1989)). For example, cells transformed with ROR are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

Cell proliferation may also be assayed by measuring ATP levels as indicator of metabolically active cells. Such assays are commercially available, for example Cell  
10 Titer-Glo<sup>™</sup>, which is a luminescent homogeneous assay available from Promega.

Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray JW et al. (1986) Int J Radiat Biol Relat Stud Phys Chem Med 49:237-55). Cells transfected with an ROR may be stained with propidium iodide and evaluated in a flow cytometer (available from Becton Dickinson), which indicates accumulation of cells in  
15 different stages of the cell cycle.

Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses an ROR, and that optionally has defective p21 function (e.g. p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no  
20 test agent is added, identify candidate p21 modulating agents. In some embodiments of the invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate p21 modulating agents that is initially identified using another assay system such as a cell-free assay system. A cell proliferation assay may also be used to test whether ROR function plays a direct role in cell proliferation or cell cycle. For example, a  
25 cell proliferation or cell cycle assay may be performed on cells that over- or under-express ROR relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the ROR plays a direct role in cell proliferation or cell cycle.

**Angiogenesis.** Angiogenesis may be assayed using various human endothelial cell  
30 systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and

tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses an ROR, and that optionally has defective p21 function (e.g. p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate p21 modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate p21 modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether ROR function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express ROR relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the ROR plays a direct role in angiogenesis. U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434, among others, describe various angiogenesis assays.

**Hypoxic induction.** The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glycolytic enzymes and VEGF. Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with ROR in hypoxic conditions (such as with 0.1% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses an ROR, and that optionally has defective p21 function (e.g. p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate p21 modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate p21 modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether ROR function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express ROR relative to wild type cells. Differences in hypoxic response compared to wild type cells suggests that the ROR plays a direct role in hypoxic induction.

**Cell adhesion.** Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents. Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2× final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF, and allowed to adhere to the monolayers in the presence of candidate agents. Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., Bioconjug Chem. 2001 May-Jun;12(3):346-53).

**Tubulogenesis.** Tubulogenesis assays monitor the ability of cultured cells, generally endothelial cells, to form tubular structures on a matrix substrate, which generally simulates the environment of the extracellular matrix. Exemplary substrates include Matrigel™ (Becton Dickinson), an extract of basement membrane proteins containing laminin, collagen IV, and heparin sulfate proteoglycan, which is liquid at 4°C and forms a solid gel at 37°C. Other suitable matrices comprise extracellular components such as collagen, fibronectin, and/or fibrin. Cells are stimulated with a pro-angiogenic

stimulant, and their ability to form tubules is detected by imaging. Tubules can generally be detected after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Tube formation assays are well known in the art (e.g., Jones MK et al., 1999, Nature Medicine 5:1418-1423). These assays have traditionally involved

5 stimulation with serum or with the growth factors FGF or VEGF. Serum represents an undefined source of growth factors. In a preferred embodiment, the assay is performed with cells cultured in serum free medium, in order to control which process or pathway a candidate agent modulates. Moreover, we have found that different target genes respond differently to stimulation with different pro-angiogenic agents, including inflammatory  
10 angiogenic factors such as TNF- $\alpha$ . Thus, in a further preferred embodiment, a tubulogenesis assay system comprises testing an ROR's response to a variety of factors, such as FGF, VEGF, phorbol myristate acetate (PMA), TNF- $\alpha$ , ephrin, etc.

**Cell Migration.** An invasion/migration assay (also called a migration assay) tests  
15 the ability of cells to overcome a physical barrier and to migrate towards pro-angiogenic signals. Migration assays are known in the art (e.g., Paik JH et al., 2001, J Biol Chem 276:11830-11837). In a typical experimental set-up, cultured endothelial cells are seeded onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The matrix generally simulates the environment of the extracellular matrix, as  
20 described above. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic stimuli. Migration is generally assayed after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Migration is assessed as the number of  
25 cells that crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific, South San Francisco, CA), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and migration is detected using fluorescent readings, for instance using the Falcon HTS FluoroBlok (Becton Dickinson). While some migration is observed in the absence of  
30 stimulus, migration is greatly increased in response to pro-angiogenic factors. As described above, a preferred assay system for migration/invasion assays comprises testing an ROR's response to a variety of pro-angiogenic factors, including tumor angiogenic and inflammatory angiogenic agents, and culturing the cells in serum free medium.

**Sprouting assay.** A sprouting assay is a three-dimensional *in vitro* angiogenesis assay that uses a cell-number defined spheroid aggregation of endothelial cells ("spheroid"), embedded in a collagen gel-based matrix. The spheroid can serve as a starting point for the sprouting of capillary-like structures by invasion into the

extracellular matrix (termed "cell sprouting") and the subsequent formation of complex anastomosing networks (Korff and Augustin, 1999, J Cell Sci 112:3249-58). In an exemplary experimental set-up, spheroids are prepared by pipetting 400 human umbilical vein endothelial cells into individual wells of a nonadhesive 96-well plates to allow overnight spheroidal aggregation (Korff and Augustin: J Cell Biol 143: 1341-52, 1998). Spheroids are harvested and seeded in 900 $\mu$ l of methocel-collagen solution and pipetted into individual wells of a 24 well plate to allow collagen gel polymerization. Test agents are added after 30 min by pipetting 100  $\mu$ l of 10-fold concentrated working dilution of the test substances on top of the gel. Plates are incubated at 37°C for 24h. Dishes are fixed at the end of the experimental incubation period by addition of paraformaldehyde. Sprouting intensity of endothelial cells can be quantitated by an automated image analysis system to determine the cumulative sprout length per spheroid.

#### ***Primary assays for antibody modulators***

For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the ROR protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for detecting ROR-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

In some cases, screening assays described for small molecule modulators may also be used to test antibody modulators.

#### ***Primary assays for nucleic acid modulators***

For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance ROR gene expression, preferably mRNA expression. In general, expression analysis comprises comparing ROR expression in like populations of cells (*e.g.*, two pools of cells that endogenously or recombinantly express ROR) in the presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting,

ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that ROR mRNA expression is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with specific antibodies or antisera directed against either the ROR protein or specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

In some cases, screening assays described for small molecule modulators, particularly in assay systems that involve ROR mRNA expression, may also be used to test nucleic acid modulators.

## Secondary Assays

Secondary assays may be used to further assess the activity of ROR-modulating agent identified by any of the above methods to confirm that the modulating agent affects ROR in a manner relevant to the p21 pathway. As used herein, ROR-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with ROR.

Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express ROR) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate ROR-modulating agent results in changes in the p21 pathway in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the p21 or interacting pathways.

## Cell-based assays

Cell based assays may use a mammalian cell line known to have defective p21 function such as HCT116 colon cancer cells available from American Type Culture Collection (ATCC), Manassas, VA). Cell based assays may detect endogenous p21

pathway activity or may rely on recombinant expression of p21 pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

5

### *Animal Assays*

A variety of non-human animal models of normal or defective p21 pathway may be used to test candidate ROR modulators. Models for defective p21 pathway typically use genetically modified animals that have been engineered to mis-express (*e.g.*, over-  
10 express or lack expression in) genes involved in the p21 pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

In a preferred embodiment, p21 pathway activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal p21 are  
15 used to test the candidate modulator's affect on ROR in Matrigel® assays. Matrigel® is an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4°C, but rapidly forms a solid gel at 37°C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells which over-express the ROR. The  
20 mixture is then injected subcutaneously(SC) into female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral (PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit). Hemoglobin  
25 content of the gel is found to correlate the degree of neovascularization in the gel.

In another preferred embodiment, the effect of the candidate modulator on ROR is assessed via tumorigenicity assays. Tumor xenograft assays are known in the art (see, *e.g.*, Ogawa K et al., 2000, *Oncogene* 19:6043-6052). Xenografts are typically implanted SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-  
30 existing tumor or from *in vitro* culture. The tumors which express the ROR endogenously are injected in the flank,  $1 \times 10^5$  to  $1 \times 10^7$  cells per mouse in a volume of 100  $\mu$ L using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100 mg. Candidate modulator is delivered IV, SC, IP, or PO by bolus administration.

Depending upon the pharmacokinetics of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors maybe  
5 utilized for biomarker identification or further analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

In another preferred embodiment, tumorigenicity is monitored using a hollow fiber  
10 assay, which is described in U.S. Pat No. US 5,698,413. Briefly, the method comprises implanting into a laboratory animal a biocompatible, semi-permeable encapsulation device containing target cells, treating the laboratory animal with a candidate modulating agent, and evaluating the target cells for reaction to the candidate modulator. Implanted cells are generally human cells from a pre-existing tumor or a tumor cell line. After an appropriate  
15 period of time, generally around six days, the implanted samples are harvested for evaluation of the candidate modulator. Tumorigenicity and modulator efficacy may be evaluated by assaying the quantity of viable cells present in the macrocapsule, which can be determined by tests known in the art, for example, MTT dye conversion assay, neutral red dye uptake, trypan blue staining, viable cell counts, the number of colonies formed in  
20 soft agar, the capacity of the cells to recover and replicate in vitro, etc.

In another preferred embodiment, a tumorigenicity assay use a transgenic animal, usually a mouse, carrying a dominant oncogene or tumor suppressor gene knockout under the control of tissue specific regulatory sequences; these assays are generally referred to as transgenic tumor assays. In a preferred application, tumor development in the transgenic  
25 model is well characterized or is controlled. In an exemplary model, the "RIP1-Tag2" transgene, comprising the SV40 large T-antigen oncogene under control of the insulin gene regulatory regions is expressed in pancreatic beta cells and results in islet cell carcinomas (Hanahan D, 1985, Nature 315:115-122; Parangi S et al, 1996, Proc Natl Acad Sci USA 93: 2002-2007; Bergers G et al, 1999, Science 284:808-812). An "angiogenic  
30 switch," occurs at approximately five weeks, as normally quiescent capillaries in a subset of hyperproliferative islets become angiogenic. The RIP1-TAG2 mice die by age 14 weeks. Candidate modulators may be administered at a variety of stages, including just prior to the angiogenic switch (e.g., for a model of tumor prevention), during the growth of small tumors (e.g., for a model of intervention), or during the growth of large and/or



invasive tumors (e.g., for a model of regression). Tumorigenicity and modulator efficacy can be evaluating life-span extension and/or tumor characteristics, including number of tumors, tumor size, tumor morphology, vessel density, apoptotic index, etc.

## 5 Diagnostic and therapeutic uses

Specific ROR-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the p21 pathway, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating the p21 pathway in a cell, preferably a  
10 cell pre-determined to have defective or impaired p21 function (e.g. due to overexpression, underexpression, or misexpression of p21, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates ROR activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the p21 function is restored. The phrase "function is restored", and equivalents, as used  
15 herein, means that the desired phenotype is achieved, or is brought closer to normal compared to untreated cells. For example, with restored p21 function, cell proliferation and/or progression through cell cycle may normalize, or be brought closer to normal relative to untreated cells. The invention also provides methods for treating disorders or disease associated with impaired p21 function by administering a therapeutically effective  
20 amount of an ROR -modulating agent that modulates the p21 pathway. The invention further provides methods for modulating ROR function in a cell, preferably a cell pre-determined to have defective or impaired ROR function, by administering an ROR -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired ROR function by administering a therapeutically  
25 effective amount of an ROR -modulating agent.

The discovery that ROR is implicated in p21 pathway provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in the p21 pathway and for the identification of subjects having a predisposition to such diseases and disorders.

30 Various expression analysis methods can be used to diagnose whether ROR expression occurs in a particular sample, including Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR, and microarray analysis. (e.g., Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann

Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-47). Tissues having a disease or disorder implicating defective p21 signaling that express an ROR, are identified as amenable to treatment with an ROR modulating agent. In a preferred application, the p21 defective tissue overexpresses an ROR relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial ROR cDNA sequences as probes, can determine whether particular tumors express or overexpress ROR. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of ROR expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

Various other diagnostic methods may be performed, for example, utilizing reagents such as the ROR oligonucleotides, and antibodies directed against an ROR, as described above for: (1) the detection of the presence of ROR gene mutations, or the detection of either over- or under-expression of ROR mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of ROR gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by ROR.

Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease or disorder in a patient that is associated with alterations in ROR expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for ROR expression; c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of the disease or disorder. Preferably, the disease is cancer, most preferably a cancer as shown in TABLE 1. The probe may be either DNA or protein, including an antibody.

## EXAMPLES

The following experimental section and examples are offered by way of illustration and not by way of limitation.

### I. *Drosophila* p21 screen

A dominant loss of function screen was carried out in *Drosophila* to identify genes that interact with the cyclin dependent kinase inhibitor, p21 (Bourne HR, et al., Nature (1990) 348(6297):125-132; Marshall CJ, Trends Genet (1991) 7(3):91-95). Expression of the p21 gene from GMR-p21 transgene (Hay, B. A., et al. (1994) Development 120:2121-

2129) in the eye causes deterioration of normal eye morphology, resulting in reduced, rough eyes. Flies carrying this transgene were maintained as a stock (P 1025 F, genotype: y w; P{p21-pExp-gl-w[+]Hsp70(3'UTR)-5}). Females of this stock were crossed to a collection of males carrying piggyBac insertions (Fraser M *et al.*, Virology (1985)

145:356-361). Resulting progeny carrying both the transgene and transposons were scored for the effect of the transposon on the eye phenotype, i.e. whether the transposon enhanced or suppressed (or had no effect) the eye phenotype. All data was recorded and all modifiers were retested with a repeat of the original cross, and the retests were scored at least twice. Modifiers of the eye phenotype were identified as members of the p21 pathway. HR46 was an enhancer of the eye phenotype. Orthologs of the modifiers are referred to herein as ROR.

BLAST analysis (Altschul et al., *supra*) was employed to identify orthologs of *Drosophila* modifiers. For example, representative sequences from ROR, GI#s 19743901, 19743907, and 19743909 (SEQ ID NOs:16, 17, and 18, respectively) share 37%, 39%, and 36% amino acid identity, respectively, with the *Drosophila* HR46.

Various domains, signals, and functional subunits in proteins were analyzed using the PSORT (Nakai K., and Horton P., Trends Biochem Sci, 1999, 24:34-6; Kenta Nakai, Protein sorting signals and prediction of subcellular localization, Adv. Protein Chem. 54, 277-344 (2000)), PFAM (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2), SMART (Ponting CP, et al., SMART: identification and annotation of domains from signaling and extracellular protein sequences. Nucleic Acids Res. 1999 Jan 1;27(1):229-32), TM-HMM (Erik L.L. Sonnhammer, Gunnar von Heijne, and Anders Krogh: A hidden Markov model for predicting transmembrane helices in protein sequences. In Proc. of Sixth Int. Conf. on Intelligent Systems for Molecular Biology, p 175-182 Ed J. Glasgow, T. Littlejohn, F. Major, R. Lathrop, D. Sankoff, and C. Sensen Menlo Park, CA: AAAI Press, 1998), and clust (Remm M, and Sonnhammer E. Classification of transmembrane protein families in the *Caenorhabditis elegans* genome and identification of human orthologs. Genome Res. 2000 Nov;10(11):1679-89) programs. For example, the zinc finger domain (PFAM 00105) of ROR from GI#s 19743901, 19743907, and 19743909 (SEQ ID NOs:16, 17, and 18, respectively) is located respectively at approximately amino acid residues 104 to 179, 8 to 83, and 29 to 104. Likewise, the Ligand-binding domain of nuclear hormone receptor domain (PFAM 00104) of SEQ ID NOs:16, 17, and 18 is located respectively at approximately amino acid residues 361 to 544, 267 to 450, and 325 to 506.

## II. High-Throughput In Vitro Fluorescence Polarization Assay

Fluorescently-labeled ROR peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of ROR activity.

## III. High-Throughput In Vitro Binding Assay.

<sup>33</sup>P-labeled ROR peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl<sub>2</sub>, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate p21 modulating agents.

## IV. Immunoprecipitations and Immunoblotting

For coprecipitation of transfected proteins,  $3 \times 10^6$  appropriate recombinant cells containing the ROR proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at  $15,000 \times g$  for 15 min. The cell lysate is incubated with 25  $\mu$ l of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase

coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

#### V. Expression analysis

5 All cell lines used in the following experiments are NCI (National Cancer Institute) lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues were obtained from Impath, UC Davis, Clontech, Stratagene, Ardaïs, Genome Collaborative, and Ambion.

10 TaqMan analysis was used to assess expression levels of the disclosed genes in various samples.

RNA was extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/μl. Single stranded cDNA was then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of  
15 Applied Biosystems (Foster City, CA).

Primers for expression analysis using TaqMan assay (Applied Biosystems, Foster City, CA) were prepared according to the TaqMan protocols, and the following criteria: a) primer pairs were designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product. Expression analysis was performed using a  
20 7900HT instrument.

Taqman reactions were carried out following manufacturer's protocols, in 25 μl total volume for 96-well plates and 10 μl total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis was prepared using a universal pool of human cDNA samples, which is a  
25 mixture of cDNAs from a wide variety of tissues so that the chance that a target will be present in appreciable amounts is good. The raw data were normalized using 18S rRNA (universally expressed in all tissues and cells).

For each expression analysis, tumor tissue samples were compared with matched normal tissues from the same patient. A gene was considered overexpressed in a tumor  
30 when the level of expression of the gene was 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue was not available, a universal pool of cDNA samples was used instead. In these cases, a gene was considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the average of all normal samples from the same tissue type was greater

than 2 times the standard deviation of all normal samples (i.e., Tumor – average(all normal samples) > 2 x STDEV(all normal samples) ).

Results are shown in Table 1. Number of pairs of tumor samples and matched normal tissue from the same patient are shown for each tumor type. Percentage of the samples with at least two-fold overexpression for each tumor type is provided. A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression analysis as described above, by antibody directed to the gene target, or by any other available detection method.

Table 1

ROR GI#	19743899	19743906	21594879
SEQ ID NO	1	8	14
Breast	3%	10%	39%
# of Pairs	30	30	36
Colon	11%	22%	24%
# of Pairs	36	36	41
Head And Neck	8%	23%	0%
# of Pairs	13	13	13
Kidney	9%	14%	27%
# of Pairs	22	22	22
Liver	0%	40%	20%
# of Pairs	5	5	5
Lung	6%	9%	7%
# of Pairs	35	35	41
Lymphoma	0%	0%	0%
# of Pairs	3	3	3
Ovary	5%	22%	63%
# of Pairs	19	18	19
Pancreas	33%	56%	33%
# of Pairs	9	9	9
Prostate	7%	7%	14%
# of Pairs	15	15	22
Skin	0%	0%	0%
# of Pairs	7	7	7
Stomach	9%	18%	0%

# of Pairs	11	11	11
Testis	0%	0%	12%
# of Pairs	8	8	8
Thyroid Gland	7%	0%	14%
# of Pairs	14	14	14
Uterus	0%	5%	43%
# of Pairs	19	19	21

## VI. ROR functional assays

RNAi experiments were carried out to knock down expression of ROR (SEQ ID NOs: 1, 8, and 14) in various cell lines using small interfering RNAs (siRNA, Elbashir et al, *supra*).

Effect of ROR RNAi on cell proliferation and growth. BrdU, Cell Titer-Glo™, and MTS assays, as described above, were employed to study the effects of decreased ROR expression on cell proliferation. The results of these experiments indicated that: RNAi of SEQ ID NO:1 decreased proliferation in 231T (a variant of MDA 231) and MDA 231 breast cancer lines (with possible mitotic arrest in 231T cells), and A549 and LX1 lung cancer lines; RNAi of SEQ ID NO:8 decreased proliferation in LX1 lung cancer line and MCF7 breast cancer line; RNAi of SEQ ID NO:14 decreased proliferation in 231T, MDA231 and MCF7 breast cancer lines, LX1 and A549 lung cancer lines, and SW480 and HCT116 colon cancer lines.

Standard colony growth assays, as described above, were employed to study the effects of decreased ROR expression on cell growth. RNAi of SEQ ID NO:1 caused inhibition of cell growth in A549, LX1, and MDA 231 cells. RNAi of SEQ ID NO:8 caused inhibition of cell growth in A549, LX1, and MDA 231 cells. RNAi of SEQ ID NO:14 did not have an affect on cell growth.

Effect of ROR RNAi on apoptosis. Nucleosome ELISA apoptosis assay, as described above, was employed to study the effects of decreased ROR expression on apoptosis. RNAi of SEQ ID NO:1 increased apoptosis in LX1 and A549 cells; RNAi of SEQ ID NO:8 increased apoptosis in LX1 cells; and RNAi of ROR SEQ ID NO:14 increased apoptosis in LX1 and A549 cells.

ROR overexpression analysis. ROR sequences were overexpressed and tested in colony growth assays as described above. Overexpression of SEQ ID NO:8 had no morphological effects on cells, and moderate effects on colony growth. Overexpression of SEQ ID NO:14 had no morphological effects on cells, and moderate effects on colony growth. Effects of overexpression of SEQ ID NO:14 on expression of various

transcription factors was also studied. Overexpression SEQ ID NO:14 caused an increased expression of the following transcription factors: SRE, AP1, ETS1, RAR $\alpha$  (DR5) and ER.



## WHAT IS CLAIMED IS:

1. A method of identifying a candidate p21 pathway modulating agent, said method comprising the steps of:

5       (a) providing an assay system comprising a ROR polypeptide or nucleic acid;  
      (b) contacting the assay system with a test agent under conditions whereby, but for the presence of the test agent, the system provides a reference activity; and  
      (c) detecting a test agent-biased activity of the assay system, wherein a difference  
10       between the test agent-biased activity and the reference activity identifies the test agent as  
a candidate p21 pathway modulating agent.

2. The method of Claim 1 wherein the assay system comprises cultured cells that express the ROR polypeptide.

15   3. The method of Claim 2 wherein the cultured cells additionally have defective p21 function.

4. The method of Claim 1 wherein the assay system includes a screening assay comprising a ROR polypeptide, and the candidate test agent is a small molecule  
20       modulator.

5. The method of Claim 4 wherein the assay is a binding assay.

6. The method of Claim 1 wherein the assay system is selected from the group consisting  
25       of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay system, and a hypoxic induction assay system.

7. The method of Claim 1 wherein the assay system includes a binding assay comprising a ROR polypeptide and the candidate test agent is an antibody.  
30

8. The method of Claim 1 wherein the assay system includes an expression assay comprising a ROR nucleic acid and the candidate test agent is a nucleic acid modulator.

9. The method of Claim 8 wherein the nucleic acid modulator is an antisense oligomer.

10. The method of Claim 8 wherein the nucleic acid modulator is a PMO.

11. The method of Claim 1 additionally comprising:

5 (d) administering the candidate p21 pathway modulating agent identified in (c) to a model system comprising cells defective in p21 function and, detecting a phenotypic change in the model system that indicates that the p21 function is restored.

12. The method of Claim 11 wherein the model system is a mouse model with defective p21 function.

10

13. A method for modulating a p21 pathway of a cell comprising contacting a cell defective in p21 function with a candidate modulator that specifically binds to a ROR polypeptide, whereby p21 function is restored.

15 14. The method of Claim 13 wherein the candidate modulator is administered to a vertebrate animal predetermined to have a disease or disorder resulting from a defect in p21 function.

20 15. The method of Claim 13 wherein the candidate modulator is selected from the group consisting of an antibody and a small molecule.

16. The method of Claim 1, comprising the additional steps of:

(e) providing a secondary assay system comprising cultured cells or a non-human animal expressing ROR ,

25 (f) contacting the secondary assay system with the test agent of (b) or an agent derived therefrom under conditions whereby, but for the presence of the test agent or agent derived therefrom, the system provides a reference activity; and

(g) detecting an agent-biased activity of the second assay system,  
wherein a difference between the agent-biased activity and the reference activity of  
30 the second assay system confirms the test agent or agent derived therefrom as a candidate p21 pathway modulating agent,

and wherein the second assay detects an agent-biased change in the p21 pathway.

17. The method of Claim 16 wherein the secondary assay system comprises cultured cells.

18. The method of Claim 16 wherein the secondary assay system comprises a non-human  
5 animal.

19. The method of Claim 18 wherein the non-human animal mis-expresses a p21 pathway gene.

10 20. A method of modulating p21 pathway in a mammalian cell comprising contacting the cell with an agent that specifically binds a ROR polypeptide or nucleic acid.

21. The method of Claim 20 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with the p21 pathway.

15

22. The method of Claim 20 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.

23. A method for diagnosing a disease in a patient comprising:

20

- (a) obtaining a biological sample from the patient;
- (b) contacting the sample with a probe for ROR expression;
- (c) comparing results from step (b) with a control;
- (d) determining whether step (c) indicates a likelihood of disease.

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24. The method of Claim 23 wherein said disease is cancer.

25. The method according to Claim 24, wherein said cancer is a cancer as shown in Table 1 as having >25% expression level.

30

## SEQUENCE LISTING

&lt;110&gt; EXELIXIS, INC.

&lt;120&gt; RORs AS MODIFIERS OF THE p21 PATHWAY AND METHODS OF USE

&lt;130&gt; EX03-068C-PC

&lt;150&gt; US 60/411,010

&lt;151&gt; 2002-09-16

&lt;160&gt; 18

&lt;170&gt; PatentIn version 3.2

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 Gln Lys Asn Cys Leu Ile Asp Arg Thr Ser Arg Asn Arg Cys Gln His  
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                                     165                                      170                                      175  
 Lys Phe Gly Arg Met Ser Lys Lys Gln Arg Asp Ser Leu Tyr Ala Glu  
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 Val Gln Lys His Arg Met Gln Gln Gln Gln Arg Asp His Gln Gln Gln  
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 Gly Leu Thr Glu Leu His Asp Asp Leu Ser Asn Tyr Ile Asp Gly His  
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 Thr Pro Glu Gly Ser Lys Ala Asp Ser Ala Val Ser Ser Phe Tyr Leu  
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 Asp Ile Gln Pro Ser Pro Asp Gln Ser Gly Leu Asp Ile Asn Gly Ile  
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 Lys Pro Glu Pro Ile Cys Asp Tyr Thr Pro Ala Ser Gly Phe Phe Pro  
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Cys Gln Tyr Leu Arg Glu Glu Leu Gln Gln Ile Thr Trp Gln Thr Phe  
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Leu Gln Glu Glu Ile Glu Asn Tyr Gln Asn Lys Gln Arg Glu Val Met  
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Trp Gln Leu Cys Ala Ile Lys Ile Thr Glu Ala Ile Gln Tyr Val Val  
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Glu Phe Ala Lys Arg Ile Asp Gly Phe Met Glu Leu Cys Gln Asn Asp  
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Gln Ile Val Leu Leu Lys Ala Gly Ser Leu Glu Val Val Phe Ile Arg  
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Met Cys Arg Ala Phe Asp Ser Gln Asn Asn Thr Val Tyr Phe Asp Gly  
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Lys Tyr Ala Ser Pro Asp Val Phe Lys Ser Leu Gly Cys Glu Asp Phe  
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Ile Ser Phe Val Phe Glu Phe Gly Lys Ser Leu Cys Ser Met His Leu  
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Thr Glu Asp Glu Ile Ala Leu Phe Ser Ala Phe Val Leu Met Ser Ala  
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Asp Arg Ser Trp Leu Gln Glu Lys Val Lys Ile Glu Lys Leu Gln Gln  
 465 470 475 480

Lys Ile Gln Leu Ala Leu Gln His Val Leu Gln Lys Asn His Arg Glu  
 485 490 495

Asp Gly Ile Leu Thr Lys Leu Ile Cys Lys Val Ser Thr Leu Arg Ala  
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Leu Cys Gly Arg His Thr Glu Lys Leu Met Ala Phe Lys Ala Ile Tyr  
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 <213> Homo sapiens

<400> 17

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Gln Arg Asn Cys Leu Ile Asp Arg Thr Asn Arg Asn Arg Cys Gln His  
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Cys Arg Leu Gln Lys Cys Leu Ala Leu Gly Met Ser Arg Asp Ala Val  
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Lys Phe Gly Arg Met Ser Lys Lys Gln Arg Asp Ser Leu Tyr Ala Glu  
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Val Gln Lys His Gln Gln Arg Leu Gln Glu Gln Arg Gln Gln Gln Ser  
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Gly Glu Ala Glu Ala Leu Ala Arg Val Tyr Ser Ser Ser Ile Ser Asn  
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Gly Leu Ser Asn Leu Asn Asn Glu Thr Ser Gly Thr Tyr Ala Asn Gly  
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His Val Ile Asp Leu Pro Lys Ser Glu Gly Tyr Tyr Asn Val Asp Ser  
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Gly Gln Pro Ser Pro Asp Gln Ser Gly Leu Asp Met Thr Gly Ile Lys  
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Gln Ile Lys Gln Glu Pro Ile Tyr Asp Leu Thr Ser Val Pro Asn Leu  
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Phe Thr Tyr Ser Ser Phe Asn Asn Gly Gln Leu Ala Pro Gly Ile Thr  
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Met Thr Glu Ile Asp Arg Ile Ala Gln Asn Ile Ile Lys Ser His Leu  
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Glu Thr Cys Gln Tyr Thr Met Glu Glu Leu His Gln Leu Ala Trp Gln  
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Thr His Thr Tyr Glu Glu Ile Lys Ala Tyr Gln Ser Lys Ser Arg Glu  
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Ala Leu Trp Gln Gln Cys Ala Ile Gln Ile Thr His Ala Ile Gln Tyr  
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Val Arg Met Cys Arg Ala Phe Asn Pro Leu Asn Asn Thr Val Leu Phe  
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Glu Gly Lys Tyr Gly Gly Met Gln Met Phe Lys Ala Leu Gly Ser Asp  
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Asp Leu Val Asn Glu Ala Phe Asp Phe Ala Lys Asn Leu Cys Ser Leu  
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Thr Ala Val Cys Asn Leu His Gly Glu Lys Leu Gln Val Phe Lys Gln  
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Tyr Ser Cys Thr Arg Gln Gln Asn Cys Pro Ile Asp Arg Thr Ser Arg  
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195	200	205
Gly Lys Ala Glu Gly Arg Glu Ser Phe Tyr Ser Thr Gly Ser Gln Leu 210 215 220		
Thr Pro Asp Arg Cys Gly Leu Arg Phe Glu Glu His Arg His Pro Gly 225 230 235 240		
Leu Gly Glu Leu Gly Gln Gly Pro Asp Ser Tyr Gly Ser Pro Ser Phe 245 250 255		
Arg Ser Thr Pro Glu Ala Pro Tyr Ala Ser Leu Thr Glu Ile Glu His 260 265 270		
Leu Val Gln Ser Val Cys Lys Ser Tyr Arg Glu Thr Cys Gln Leu Arg 275 280 285		
Leu Glu Asp Leu Leu Arg Gln Arg Ser Asn Ile Phe Ser Arg Glu Glu 290 295 300		
Val Thr Gly Tyr Gln Arg Lys Ser Met Trp Glu Met Trp Glu Arg Cys 305 310 315 320		
Ala His His Leu Thr Glu Ala Ile Gln Tyr Val Val Glu Phe Ala Lys 325 330 335		
Arg Leu Ser Gly Phe Met Glu Leu Cys Gln Asn Asp Gln Ile Val Leu 340 345 350		
Leu Lys Ala Gly Ala Met Glu Val Val Leu Val Arg Met Cys Arg Ala 355 360 365		
Tyr Asn Ala Asp Asn Arg Thr Val Phe Phe Glu Gly Lys Tyr Gly Gly 370 375 380		
Met Glu Leu Phe Arg Ala Leu Gly Cys Ser Glu Leu Ile Ser Ser Ile 385 390 395 400		
Phe Asp Phe Ser His Ser Leu Ser Ala Leu His Phe Ser Glu Asp Glu 405 410 415		
Ile Ala Leu Tyr Thr Ala Leu Val Leu Ile Asn Ala His Arg Pro Gly 420 425 430		
Leu Gln Glu Lys Arg Lys Val Glu Gln Leu Gln Tyr Asn Leu Glu Leu 435 440 445		

Ala Phe His His His Leu Cys Lys Thr His Arg Gln Ser Ile Leu Ala  
450 455 460

Lys Leu Pro Pro Lys Gly Lys Leu Arg Ser Leu Cys Ser Gln His Val  
465 470 475 480

Glu Arg Leu Gln Ile Phe Gln His Leu His Pro Ile Val Val Gln Ala  
485 490 495

Ala Phe Pro Pro Leu Tyr Lys Glu Leu Phe Ser Thr Glu Thr Glu Ser  
500 505 510

Pro Val Gly Leu Ser Lys  
515